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Mol. Cell. Biol. 1992, 12(8), 3636–3643
Virology 1992, 186(2), 669–675
J. Virology 1988, 62(11), 4059–4069

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(54) Bicistronic RNA viruses expressing heterologous polypeptide

(57) A eukaryotic RNA virus, especially a picornavirus and, most especially, a poliovirus, which is made bicistronic with respect to its RNA function and is able to replicate and to express a foreign polypeptide in cells infected with the virus, is described. The virus may have a genome provided in order (5' to 3') with a first ribosome landing pad (RLP) operably linked to a first gene, a translational stop codon, and a second RLP, functionally independent of the first RLP and operably linked to a second gene, one of said genes being native to the virus and the other being a foreign gene capable of expressing a foreign polypeptide, and one of said RLPs being native and the other being foreign.

GB 2 262 099

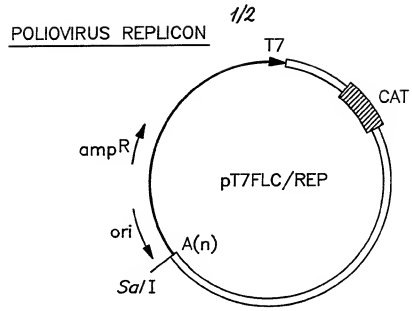

POLIOVIRUS REPLICON
T7
CAT
ampR
pT7FLC/REP
ori
A(n)
Sal I
RNA:
5'NCR
CAT
ΔP1
P2
P3
3'NCR
A(n)
AUG
in-frame fusion

Fig.1

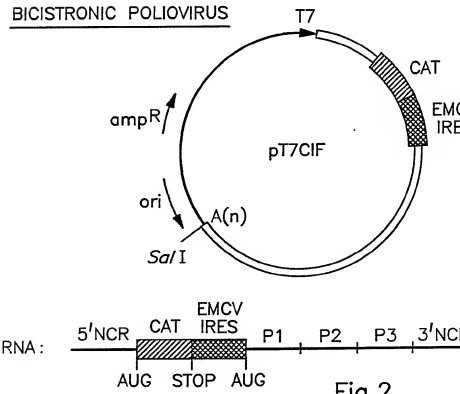
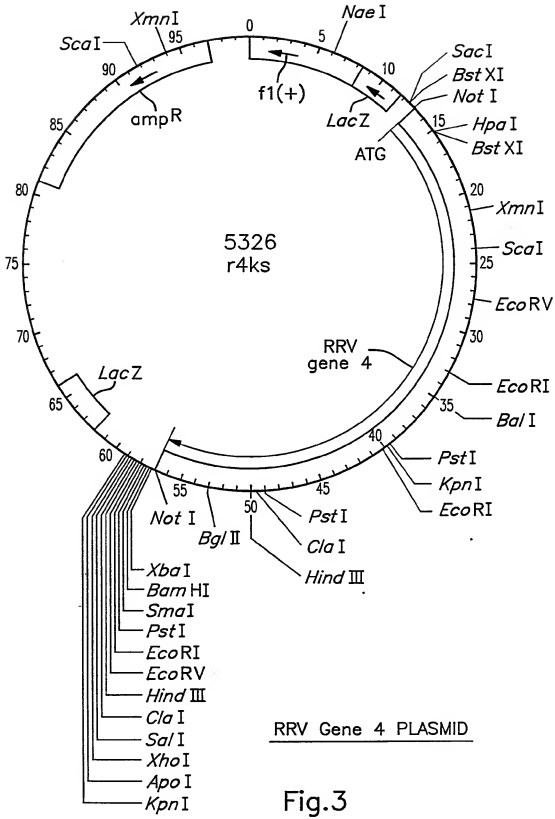

BICISTRONIC POLIOVIRUS
T7
CAT
EMCV IRES
ampR
pT7CIF
ori
A(n)
Sal I
RNA:
5'NCR
CAT
EMCV IRES
P1
P2
P3
3'NCR
A(n)
AUG
STOP
AUG

Fig.2



RRV Gene 4 PLASMID

Fig.3

BICISTRONIC VIRUSES

Background of the invention

1. Field of the invention

The invention relates to the expression of foreign polypeptides.

2. Description of the related art

Poliovirus is a single-stranded RNA virus. The positive sense genome comprises a 5' non-coding region of about 750 nucleotides, a single open reading frame and a short 3' non-coding region followed by a poly-adenine tail. The open reading frame is translated as a single polypeptide which is post-translationally cleaved by viral encoded proteases into the viral structural and non-structural proteins.

Defective interfering genomes (DIs) of poliovirus arise during propagation of virus at high multiplicities in tissue culture. These genomes retain all essential sequences for replication but lack some non-essential sequences. Therefore they are not infectious alone but can be propagated by superinfecting with helper virus which provides the deleted functions in trans. It has been noted that the deletions in naturally-occurring DIs map to the P1 region of the genome which encodes the viral structural proteins (Nomoto et al., J. Mol. Biol. 128, 179-196, 1979). Artificial DIs can be constructed by deleting regions of P1 (Hagino-Yamagishi and Nomoto, J. Virol. 63, 5386-5392, 1989). It has recently been shown that foreign sequences can replace those non-essential regions of P1. In these cases the foreign sequences are replicated by the poliovirus polymerase and expressed in transfected cells (Choi et al., J. Virol. 65, 2875-2883, 1991).

It is a problem to find some means of expressing polypeptides from viruses especially picornaviruses without using a helper virus.

Summary of the invention

The present invention provides a eukaryotic RNA virus which is made bicistronic with respect to its RNA function and is able to replicate and to express a foreign polypeptide in cells

infected with the virus. The virus, in addition to carrying its own genetic information, also carries a foreign gene which can be independently expressed in a cell infected with the virus. The resulting foreign polypeptide is a distinct entity unattached to any viral proteins, and would not normally form part of the viral particle.

The invention also includes the preparation of a bicistronic virus by constructing a DNA sequence which corresponds to the genome of the bicistronic RNA virus and obtaining live virus from the DNA sequence this constructed. It also includes replicating the virus, eukaryotic cells infected with the virus and a vaccine based thereon and also the use of the virus for producing a polypeptide by culturing infected eukaryotic cells.

A consequence of poliovirus infection is that host cell protein synthesis is shut off due to inactivation of the CAP-binding complex, part of the normal translation machinery of the cell. Thus, poliovirus itself must employ a CAP-independent mode of translation. The cis-acting elements responsible for this have been mapped to the 5' non-coding region between nucleotides 130 and 620. Indeed, insertion of these sequences upstream of a reporter gene can confer CAP-independent translation of "an artificially created mRNA" (actually a DNA construct) (Pelletier and Sonenberg, Nature 334, 320-325, 1988).

Additional description of the prior art

The sequences responsible have been more precisely defined by Nicholson et al., J. Virology 65, 5886-5894 (1991) and termed the "ribosomal landing pad (RLP).

The initiation of translation by an internal ribosome-binding mechanism, in which the CAP structure is by-passed, is known in encephalomyocarditis virus (EMCV), Jang et al., J. Virology 63, 1651-1660 (1989) and in foot and mouth disease virus (FMDV), Kahn et al., J. Virology 64, 4625-4631. Macejak and Sarnow, Nature 353, 90-94 (1991) confirm the observations of Pelletier and Sonenberg supra with respect to the poliovirus RLP and report also that a 5' leader sequence of a cellular mRNA "BiP" (human Ig heavy-chain binding protein) contains an internal ribosome-binding

site and suggest that other (unspecified) cellular mRNAs might also possess one. These papers describe DNA constructs having in order (5' to 3') a conventional promoter, a first gene, a stop codon, a RLP and a second gene. These bicistronic constructs were made to demonstrate the independent expression of the second gene. It was not suggested in the above-mentioned papers that such constructs have any practical use. They differ from those of the present invention in that they do not contain viral genomic sequence containing a native viral RLP, nor any downstream native viral gene and, in particular, are incapable of generating viral particles. The present invention thus provides an entirely new form of viral vector.

Palmenberg et al., U.S. Patent 4,937,190, disclose a recombinant DNA vector comprising a promoter, a RLP from a cardiovirus and a foreign gene, the RLP being described as an enhancer of the translation of RNA obtainable from DNA sequences. However, the use of two RLPs and genes and the production of viral particles is not disclosed.

Many viruses are capable of generating a bicistronic mRNA, in the sense that two genes are transcribed from a single promoter acting on consecutive or overlapping open reading frames. It will be appreciated that such viruses are not bicistronic in the sense of being able to express a foreign gene and to do so independently of the native viral gene.

Brief description of the drawings

Figure 1 illustrates a plasmid containing poliovirus cDNA and a foreign (reporter) gene, used in the construction of a viral cDNA for use in comparative experiments of gene expression;

Figure 2 illustrates a plasmid containing poliovirus cDNA (with its native RLP), a foreign reporter gene, stop codon and foreign RLP, for use in the invention with its corresponding RNA transcript (see Example 1); and

Figure 3 illustrates a rhesus rotaviruses (RRV) cDNA plasmid which is used in the construction of a plasmid containing cDNA corresponding to the RNA genome of a bicistronic virus of the invention (see Example 2).

Description of the preferred embodiments

The genome of the bicistronic virus comprises in order (5' to 3') a 5' replication-initiating site, a first ribosome landing pad (RLP), operably linked to a first gene provided with a translational stop codon, and a second RLP, operably linked to the coding sequence for a second gene, one of the said genes being a native, viral, gene and the other being a foreign gene capable of expressing the foreign polypeptide, and one of the said RLPs being native to the virus and the other foreign. One cistron is therefore the foreign gene and the other cistron is a native viral gene. We have developed constructs in which the first gene is the foreign gene and the second is a viral gene and, while this embodiment is described hereinafter in detail, the alternative order (in which the first gene is native, the second foreign) is also possible. In our construct the first RLP is native and the second foreign, but the reverse order is possible. There are therefore four possible orders of the RLPs and genes, i.e. (1) native RLP, native gene, foreign RLP, foreign gene; (2) native RLP, foreign gene, foreign RLP, native gene; (3) foreign RLP, native gene, native RLP, foreign gene; (4) foreign RLP, foreign gene, native RLP, native gene. Accordingly this detailed description applies mutatis mutandis to these alternative possibilities. The bicistronic virus remains replication-competent and does not require helper viruses or helper cell cultures for propagation.

The virus may be a positive-strand RNA virus, a negative-strand RNA virus or a double-strand RNA virus. Preferably the virus is a positive-strand RNA virus. It may be a picornavirus such as an enterovirus, cardiovirus e.g. EMCV, aphthovirus e.g. FMDV, or rhinovirus, an alpha virus, a flavi virus, or a corona virus. The virus may be a plant virus such as a comovirus, cucomovirus, bromovirus or mosaic virus, especially tobacco mosaic virus.

It is particularly preferred that the RNA virus is a poliovirus, for example a type 1, type 2 or type 3 poliovirus or a coxsackie virus. The poliovirus is preferably attenuated, but may

be neurovirulent (since a killed [inactive] viral preparation can be made). It may therefore be type 1 Sabin, type 1 Mahoney, type 2 Sabin or type 3 Leon strain, for example.

A replication-initiating site is located at the 5'-end of the genome of the bicistronic virus. The genome is therefore provided with a polymerase recognition site for the purpose of enabling the virus to replicate. Typically the replication-initiating site is the native such site for the virus. The native viral polymerase encoded by the viral coding sequence can therefore initiate replication at the 5' replication-initiating site. In the case of bicistronic poliovirus, the 5' end of the genome of the virus should consist of the native poliovirus polymerase binding sequence at which the poliovirus RNA-dependent RNA polymerase is able to initiate replication.

The first (RLP) is provided downstream of the 5' replication initiating site. An alternative term for a RLP is an internal ribosome entry site (IRES). The term RLP is usually used in connection with poliovirus. The term IRES is usually used in connection with encephalomyocarditis virus (EMCV). The RLP enables internal initiation of translation to occur. Translation of the foreign gene provided in the genome of the bicistronic virus can therefore occur in a CAP-independent fashion.

A virus may have its own RLP In the 5' non-coding region (NCR) of the viral genome. Poliovirus is an example of a picornavirus which has its own RLP in the 5' NCR of the viral genome. In such circumstances, therefore, the native 5' NCR of the virus or at least the portion of the 5' NCR essential for virus viability is preferably present to provide the 5' replication initiating site and the first RLP of the bicistronic virus. It has previously been reported, for example, that the sequences in the downstream region of the 5' NCR of the poliovirus genome are dispensible for viability in tissue cultures (Iizuka et al, J. Virol. 63, 5354-5363, 1989). A bicistronic poliovirus may therefore incorporate in its genome the native poliovirus 5' NCR lacking nucleotides 673 to 743.

In one embodiment of the invention a virus, a bicistronic picornavirus, such as a poliovirus, may therefore be constructed in which a foreign gene and a foreign RLP are provided in that order in the viral genome between the 5' NCR, or at least the portion thereof essential for virus viability or growth, and the protein-coding region of the genome. Either or both of the foreign gene and the foreign RLP can be inserted within a non-essential region of the 5' NCR or between the 3' end of the 5' NCR and the start codon for the viral gene. The first native, RLP is operably linked to the foreign gene. The polypeptide encoded by the foreign gene can thus be translated. The RLP has a translational start codon, immediately downstream of which is located the foreign gene. Internal initiation of translation can therefore take place so that the polypeptide encoded by the foreign gene is expressed in cells infected with the bicistronic virus.

The foreign gene may encode any polypeptide which is foreign in relation to the native virus. The bicistronic virus can be used as a vaccine. The foreign gene may consequently code for an antigenic polypeptide capable of inducing either a T or a B cell response or both. The foreign gene may therefore encode an antigenic polypeptide derived from a virus, bacterium, fungus, yeast or parasite, for example. The antigenic polypeptide may be capable of raising neutralising antibody to a pathogen.

A foreign polypeptide can therefore be expressed which comprises an antigen capable of raising neutralising or non-neutralising antibody. The antigen may be derived from a human immunodeficiency virus (HIV) such as HIV-1 or HIV-2, a hepatitis virus such as hepatitis A, B or C virus, a human rhinovirus such as type 2 or type 14, herpes simplex virus (HSV), foot-and-mouth disease virus, influenza virus, coxsackie virus, the cell surface antigen CD4, Chlamydia trachomatis, plasmodium falciparum, etc. The polypeptide that is expressed may be influenza haemaglutinin, HIV gp120 or gp160 or HSV glycoprotein D, for example.

The bicistronic virus may alternatively be used for the expression of proteins, typically eukaryotic proteins, in culture. Any useful protein may therefore be encoded by the foreign gene, such as physiologically active polypeptide. A polypeptide of therapeutic use may therefore be produced in culture and isolated. The polypeptide may be calcitonin, tissue plasminogen activator, a growth factor such as human growth hormone, GM-CSF, G-CSF, etc.

A second RLP is located downstream of the translational stop codon of the forming gene. The first and second RLPs must be different, to prevent recombination occurring within the genome of the bicistronic virus. A preferred second RLP is the RLP of (Jang et al. EMCA supra). A RLP should be selected which enables a stable, viable virus to be efficiently produced. We have found that bicistronic poliovirus was not efficiently obtained when we used, as the second RLP, the 5' untranslated leader derived from the cellular message of GRP78 (BiP, Jacejak and Sarnow, Nature 353. 90-94, 1991). A certain amount of routine experimentation may therefore be required to select an appropriate second RLP.

The coding sequence for the native viral proteins is operably linked to the second RLP. The coding sequence is positioned immediately downstream of the translation start codon at which translation normally commences for the second RLP. The native viral proteins can thus be expressed. The coding sequence for the native viral proteins may then be followed by a 3' NCR, typically the native 3' NCR for the virus.

A bicistronic RNA virus is prepared by a process comprising:

a) constructing a DNA sequence which corresponds to the genome of the bicistronic RNA virus; and

b) obtaining live virus from the DNA sequence thus constructed.

A DNA sequence consisting essentially of the various elements mentioned above of the genome of the bicistronic virus is therefore assembled in step (a). The elements are ligated together as appropriate. The size of the sequences which are inserted in the native viral genome may need to be restricted to

ensure that a viable bicistronic virus is obtained. Viable virus might not be obtained where the inserted sequences are too long. For a poliovirus, for example, the inserted sequences should generally not be more than 1500 bases, for example no more than 1200 bases or 1000 bases, long.

Where a bicistronic virus is required in which the 5' replication initiating site and first RLP are provided in the form of the native 5' NCR of the virus, a DNA corresponding to the genome of the native virus may first be digested to separate the 5' NCR from the remainder of the viral genome. This can be with a single restriction enzyme at or upstream of the initiating ATG codon for the viral protein-coding sequence or with two restriction enzymes at or near this site. In the latter case a small part of the 5' non-coding sequence is replaced by the foreign sequence. Sequences in the last 100 nucleotides of the 5' NCR of a poliovirus have been shown to be dispensible for virus growth at least in tissue culture, as mentioned above. A DNA sequence, such as a cDNA sequence, encoding the foreign polypeptide it is wished to express and a DNA sequence, again such as a cDNA sequence, including a foreign RLP, may then be inserted between the 5' NCR and the remainder of the viral genome. Alternatively the foreign sequence could be inserted within the non-essential region of the 5'-NCR.

Live virus may be recovered by transfecting cells in culture with an RNA transcript of the DNA sequence which has been constructed. Live poliovirus, for example, can be recovered from a poliovirus DNA construct in which a foreign gene and a foreign RLP have been inserted by production of a positive sense RNA typically using a T7 promoter to direct transcription *in vitro* (Van der werf *et al*, 1986, Proc. Natl. Acad. Sci. USA **83**: 2330-2334). The recovered RNA can be applied to tissue cultures using standard techniques (Koch, Current Topics Microbiology and Immunology **61**: 89-138, 1973). After 4 to 6 day incubation, virus can be recovered from the supernatant of the tissue culture.

If bicistronic virus is obtained, the virus must be able to replicate. Analysis of the plaque size phenotype of the virus may

indicate how well the virus does replicate and is also a measure of the stability of the genome. A homogeneous plaque size indicates a stable genome which is not undergoing recombination events.

The bicistronic virus may also be tested for its ability to express the desired forming polypeptide. Appropriate host cells are infected with the bicistronic virus. The infected cells are cultured. The culture may then be analysed for evidence of the presence of the foreign polypeptide. Cytoplasmic extracts from the cultured cells can be analysed. Analysis techniques appropriate for each particular foreign polypeptide are employed.

The bicistronic virus may be isolated and purified. The virus may be replicated to obtain larger quantities, by infecting susceptible cells with the virus and culturing the infected cells. Since the virus is eukaryotic, i.e. one which normally replicates in eukaryotic cells, the host cells are normally eukaryotic cells. The virus can then be obtained, and isolated and purified as necessary, from the culture supernatant. Growth, assay and purification of the bicistronic virus may be as described by P. D. Minor in "Virology, a practical approach", editor B. W. Mahy, IRL Press, Oxford, GB, 1985.

The bicistronic viruses can be used in several ways. As mentioned above, they can be used as vaccines. For this purpose, an attenuated virus or a killed neurovirulent virus may be employed. The Sabin strains of poliovirus are established as effective vaccines. This coupled with the extensive experience of their manufacture and control make them a particularly attractive vector for use as a vehicle for the expression of potentially important proteins. Since poliovirus is able to induce a mucosal as well as a systemic response, the approach may be of considerable value for producing vaccines against pathogens which, like poliovirus itself, infect via a mucosal surface.

The bicistronic virus may therefore be formulated as a pharmaceutical or veterinary vaccine composition further comprising a pharmaceutically or veterinarily acceptable carrier or diluent. Any carrier or diluent conventionally used in vaccine

preparations may be employed. For example, the presently live attentuated poliovirus strains are stabilised in a solution of 1M $MgCl_2$.

The bicistronic viruses may therefore be used to prevent infections and/or diseases in a human or animal. The viruses may also be administered for therapeutic reasons. For either purpose, they may be administered orally, as a nasal spray or parenterally, for example by subcutaneous or intramuscular injection. A dose corresponding to the amount administered for a conventional live poliovirus vaccine, such as from 10^5 to $10^{6.5}TCID_{50}$, may be given although the dose will depend upon a variety of factors including the viability and replicative capacity of the virus and the purpose of administering the virus.

The bicistronic viruses can also be used as a means of producing polypeptides in culture. They may therefore be used as expression vectors for the production of foreign polypeptides in culture. Cells infected with the bicistronic virus can be cultured and the foreign polypeptide that is expressed may be obtained. The foreign polypeptide may be isolated and purified and, if desired, formulated into a pharmaceutically or veterinarily acceptable carrier or diluent.

A bicistronic poliovirus may be particularly useful for producing foreign protein in eukaryotic cells in culture, such as African green monkey cells or cells used conventionally in the production of poliovirus vaccines. Polioviruses are able to induce shut-off of host gene expression. High yields of the foreign polypeptide encoded by the bicistronic poliovirus may therefore be obtained.

The following Examples illustrate the invention.

EXAMPLE 1: CONSTRUCTION OF A BICISTRONIC POLIOVIRUS EXPRESSING THE CAT GENE

The starting material for the construction of a cDNA encoding a bicistronic virus was the plasmid pT7FLC. This contains a complete cDNA of poliovirus type 3, P3/Leon/37 (Stanway *et al*, Archives of Virology *81*, 67-68, 198) within a pBR322-based vector, except that the sequences between the ATG start codon at

nucleotide 743 and the SmaI restriction site at 2766 are derived from a clone of Sabin type 3 strain of poliovirus (Westrop et al, Journal of Virology. 63, 1338-1344, 1989) in which the SstI restriction site has been destroyed by a silent mutation. In addition, the AatII site at position 4286 in vector sequences are under the transcriptional control of the T7 promoter which has been replaced by a NotI site. The viral sequences are under the transcriptional control of the T7 promoter which has been inserted in the EcoRI site (position 0 of pBR322). The viral sequences terminate with 30 adenine residues, followed by a unique SalI restriction site which is equivalent to that at position 651 of pBR322. When linearized with SalI enzyme, this plasmid forms the template for transcription of a genome-like RNA by T7 polymerase (Van der Werf et al, 1986), and this RNA has an infectivity of 10^5 plaque-forming units (pFU) per µg when transfected into susceptible cells, for example Ohio HeLa cells.

A derivative of pT7FLC is pT7FLC/REP. In this plasmid, some of the viral sequences have been replaced with those encoding the gene for the enzyme chloramphenicol acetyl transferase (CAT). This was achieved by mutating 5' terminal and 3' terminal CAT sequences from the vector RSVCAT (Gorman et al., Mol. Cell. Biol. 2, 1044-1051, 1892) to create unique restriction enzyme sites SstI and AatII respectively. The CAT gene was then inserted into pT7FLC digested with the same unique enzymes. The structures of the fusion sites between CAT and poliovirus sequences are shown below:

```
Met Gly Ala Gln Ile Thr Gly Tyr   SEQ ID NO: 1
ATG GGA GCT CAA ATC ACT GGA TAT   SEQUENCE AT N-TERMINAL
  1             5                 POLIO-CAT FUSION

Gln Gly Gly Arg Thr Ser Arg Asn Leu  SEQ ID NO: 2
CAA GGA GGT GCG ACG TCA GAC AAC CTC  SEQUENCE AT C-TERMINAL
  1             5                    CAT-POLIO FUSION
```

The internal ribosome entry (IRES) from encephalomyocarditis virus (EMCV) was amplified using the polymerase chain reaction (PCR) from plasmid pCITE (obtained from Novagen) (Parks et al, J.

Virol 60, 376-384, 1986; Palmenberg et al. U.S. Patent 4937190) with the primers WSB70 and WSB82 whose sequences are shown below (in the 5' to 3' sense):

WSB 70 SEQ ID NO: 3

CCCGGGGAGC TCCCATATTA TCATCGTGTT TTTCAAAGG 39

SstI

WSB 82 SEQ ID NO: 4

TTCAGTGGAT CCATCGATTA AACCGCGGCG CCCCTCTCCC TCCCCCCCCC CTAA 54

BamHI ClaI SstII

The 550bp fragment obtained was cloned into pT7FLC on BamHI and SstI restriction sites (nucleotides 673 and 743 of poliovirus sequence). The resulting plasmid, pT7IRESFLC, produced an RNA after T7 transcription which gave rise to a viable virus with wild-type plaque phenotype. Nucleotides 673 to 743 of the poliovirus sequence were missing yet the virus was still fully infectious.

The primer was designed such that pT7IRESFLC would possess unique restriction sites, ClaI and SstII. Thus the plasmid can act as a cassette for the insertion of foreign sequences flanked by these restriction sites. One such sequence, that encoding the enzyme chloramphenicol acetyl-transferase (CAT), was amplified using the PCR from plasmid pT7FLC/REP, which is represented in Figure 1, with the primers WSB 80 and WSB 81 whose sequences are shown below (in the 5' to 3' sense):

WSB 80, SEQ ID NO: 5

TTCAGTATCG ATATGGAGAA AAAAATCACT GGATATACC 39

ClaI START

WSB 81 SEQ ID NO: 6

GTCGACCGCG GTTACGCACC TCCTTGCCAT TCGTCGC 37

SstI STOP

There was a TTA STOP codon at the end of the CAT gene. The product, a 660 bp fragment, was cloned into pT7IRESFLC on ClaI and SstII restriction sites. The resulting plasmid, pT7CIF, is represented in Figure 2. RNA transcribed from SalI-linearized pT7CIF gave rise to a viable virus after transfection into Ohio HeLa cells. The infectivity of the transcript was approximately

10^5 pfu per µg, i.e. the virus was as infectious as that from pT7FLC, the full length clone. However the plaques derived from pT7CIF were small as shown in Table 1.

Table 1 Plaque sizes of bicistronic viruses

Virus	Source	Plaque diameter (mm)
FLC	T7 RNA	4
CIF	T7 RNA	1
FLC	passaged virus	3
CIF	passage 1	2
CIF	passage 2	2
CIF	passage 3	2

The overall length of genomes of virus derived from pT7CIF is 8,590 nucleotides i.e. 15% longer than wild-type polioviruses.

Transfection of RNA transcribed from pT7CIF resulted in functional CAT enzyme activity, illustrated in Table 2. For comparison we show the CAT activity following transfection of a poliovirus "REP", derived from a non virus-producing replicon in which the CAT gene was inserted in place of some of the sequences encoding structural proteins (derived from plasmid pT7FLC/REP): (see above).

Table 2

RNA/virus	Time Post-infection transfection	CAT activity	
REP	3 hours	+	(comparative)
REP	6 hours	++	(" ")
REP	9 hours	++++	(" ")
CIF virus	3 hours	-	(this invention)
CIF virus	6 hours	+	(" ")
CIF virus	9 hours	+++	(" ")
CIF virus p1	3 hours	+	(" ")
CIF virus p1	6 hours	++	(" ")
CIF virus p1	9 hours	+++	(" ")
CIF virus p2	3 hours	-	(" ")

CIF virus p2	6 hours	++	(this invention)
CIF virus p2	9 hours	+++	(" ")
CIF virus p3	3 hours	-	(" ")
CIF virus p3	6 hours	++	(" ")
CIF virus p3	9 hours	+++	(" ")

The observations that transfection of RNA from pT7CIF gave rise to both viable virus and CAT enzyme activity suggests that both poliovirus and EMCV RLPs (internal entry sites) are initiating translation independently within the same genome, since there is a STOP codon between the CAT gene and the start of the poliovirus polypeptide.

Virus obtained from transfection of pT7CIF RNA was passaged three times at high multiplicity of infection (moi) through Ohio HeLa cells. The resulting viruses from each passage level were plaqued in Ohio HeLa cells. The plaques were homogeneous and their size remained constant and smaller than those of wild-type poliovirus (Table 1).

CAT assays were performed on cytoplasmic extracts from cells infected with virus from each passage (p) level. Table 2 shows that the CAT activity produced was the same at each passage, indicating that the recombinant genomes were stable on passage.

EXAMPLE 2: CONSTRUCTION OF A BICISTRONIC POLIOVIRUS EXPRESSING THE VP8 PEPTIDE FROM RHESUS ROTAVIRUS (RRV).

The VP8 cDNA from Rhesus rotavirus was amplified by PCR from plasmid r4KS (obtained from Lucia Fiore, Instituto Superiore di Sanita, Viale Regina Elena, 299, Rome), using primers of the sequence shown below (5' to 3' sense):

DS 06-0067, SEQ ID NO: 7:-
TTCAGTATCG ATATGGCTTC GCTCATTTAT AGAC 34
and DS 06-0068, SEQ ID NO: 8:-
GTCGACCGCG GTTATCTATG TGATATTATA TTTCTAGC 38

One primer (SEQ ID NO: 7) introduces a ClaI site (ATCGAT) and a START codon (ATG) at the 5' terminal of the VP8 gene, and the other introduces a STOP codon (TTA) and SstII site (CCGCGG).

Part of the + primer is derived from nucleotides 10-31 of the rhesus rotavirus VP4 gene sequence and the part of the -primer from the complement of nucleotides 748 to 727.

The polio virus plasmid pT7CIF (see Example 1) was digested with ClaI and SstII to release the CAT gene, which was replaced with the VP8 PCR product digested with Cla I and Sst II to produce pT75'VP8 IRES Leon. pT75'VP8 IRES Leon was linearised with SalI and T7 transcripts transformed into Ohio Hela cells as described for pT7CIF. This gave rise to a viable virus VP8 LEON.

This virus has been passaged twice in Ohio Hela cells and P1 and P2 checked by immuno precipitation for the expression of VP8 using a polyclonal and monoclonal antiserum to RRV. Both produced negative results.

However, the VP8 gene was amplified from progeny virus, using the primers described above, indicating the presence of the gene. It is believed that the antisera used were defective and the immunoprecipitation is being repeated.

CLAIMS

1. A eukaryotic RNA virus which is made bicistronic with respect to its RNA function and is able to replicate and to express a foreign polypeptide in cells infected with the virus.

2. A virus according to claim 1 having its genome provided in order (5' to 3') with:

a first ribosome landing pad (RLP) operably linked to a first gene, a translational stop codon and a second RLP, functionally independent of the first RLP and operably linked to a second gene, one of said genes being a native to the virus and the other being a foreign gene capable of expressing the foreign polypeptide and one of said RLPs being native to the virus and the other being foreign.

3. A virus according to claim 2 wherein the first RLP is native and the first gene is the foreign gene.

4. A virus according to claim 2 wherein the first RLP is native and the second gene is the foreign gene.

5. A virus according to claim 2, 3 or 4 which is a picornavirus.

6. A virus according to claim 5 in which the foreign RLP is of another picornavirus.

7. A virus according to claim 5 or 6 in which the picornavirus is a poliovirus.

8. A virus according to claim 7 in which the foreign RLP is of encephalomyocarditis virus (EMCV).

9. A virus according to claim 7 or 8 in which the first RLP is present within a first part of the 5'-NCR of poliovirus essential for viral growth, and the foreign gene, and the second RLP are present in that order within a second part of the 5'-NCR downstream in the 3'-direction of the first part, non-essential for viral growth, or between the 3'-end of the 5'-NCR and the start codon for the viral gene.

10. A virus according to any one of the preceding claims, in which the foreign polypeptide comprises an antigen capable of raising neutralising or non-neutralising antibody.

11. A virus according to claim 9, in which the antigen is derived from a human immunodeficiency virus, a hepatitis virus, a human rhinovirus, herpes simplex virus, foot-and-mouth disease virus, influenza virus, coxsackie virus, the cell surface antigen CD4, Chlamydia trachomatis or Plasmodium falciparum.

12. A virus according to any one of claims 1-9, in which the polypeptide is calcitonin, tissue plasminogen activator, human growth hormone, GM-CSF or G-CSF.

13. DNA which corresponds to the RNA of the bicistronic RNA virus as defined in any one of claims 1-12.

14. A process for the preparation of a bicistronic RNA virus as claimed in claim 1, which process comprises:

(a) constructing a DNA which corresponds to the RNA of the bicistronic RNA virus as defined in any one of claims 1-12; and

(b) obtaining live virus from the DNA sequence thus constructed.

15. A process for replicating a bicistronic RNA virus as claimed in any one of claims 1-12, which process comprises infecting susceptible eukaryotic cells with the virus and culturing the infected cells.

16. A vaccine comprising eukaryotic cells infected with a bicistronic RNA virus as claimed in any of claims 1-12 in which the foreign polypeptide is an antigenic polypeptide capable of inducing either a T or a B cell response or both.

17. A vaccine according to claim 16 further comprising a pharmaceutically or veterinarily acceptable carrier or diluent.

18. A process for producing a desired polypeptide, which process comprises culturing eukaryotic cells infected with a bicistronic RNA virus as claimed in claim 1 which is able to express the desired polypeptide in the said cells and obtaining the desired polypeptide.

19. A process according to claim 18, in which the desired polypeptide is a polypeptide of therapeutic use and is formulated with a pharmaceutically or veterinarily acceptable carrier or diluent.

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Databases (see over)
(i) UK Patent Office

(ii) ONLINE DATABASES: WPI, DIALOG/BIOTECH

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Documents considered relevant following a search in respect of claims 1-19

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
P X	Mal. Cell. Biol. 1992, 12(8), 3636-3643 Construction of a Bifunctional in RNA in the Mouse by Using the Internal ...	1
P X	Virology 1992, 186(2), 669-675 A Spleen Necrosis Virus-Based Retroviral Vector Which Expresses Two Genes in ...	1
A	J. Viral. 1988, 62(11), 4059-4069 Expression of Bicistronic Measles Virus P/C mRNA by Using Hybrid Adenoviruses ...	1

Category	Identity of document and relevant passages -19-	Relevant to claim(s)

Categories of documents

X: Document indicating lack of novelty or of inventive step.

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